Optimization of Prokaryotic Expression Conditions and Detection of Enzymatic Activity of Aldosene Reductase α-KAR

Chenxi Sun, Daling Liu

School of jinan University, Guangzhou 510000, China.

Abstract

α-KAR is a member of NADPH-dependent oxidoreductase superfamily. In this study, the prokaryotic expression vector was constructed and overexpressed in Escherichia coli BL21 (DE3). The highly purified and active α-KAR protein was obtained by nickel column affinity chromatography. The effects of different conditions, such as temperature, inducer concentration, induction time and culture speed, on the expression of α-KAR enzyme were studied, and the enzyme activity kinetics was analyzed. A large amount of soluble protein can be expressed at 30℃, 130rpm/h, 20 h and 0.40 m inducer concentration. The results of enzyme activity kinetics showed that p-nitrobenzaldehyde as substrate, reaction temperature 37℃, PH=7, NADPH concentration 0.2 mmol/L, substrate concentration 4.00 mmol/L enzyme activity was the best.

Keywords

Aldehyde Reductase; α-KAR; Prokaryotic Expression; Enzymatic Activity.

1. Introduction

Aldo-keto reductase (AKR;EC2.7.4.3), a monomeric NADPH-dependent oxidoreductase, is a superfamily protein composed of 190 members from 16 families [1, 2, 3], in Highly evolutionary retention in prokaryotes and eukaryotes, 320 amino acid residues, 34 to 37 kd molecular weight, more than 60% homology of proteins within subtypes, 40% homology of proteins between subtypes [4,5], including acetaldehyde Reductase (aldehyde reductase), aldose reductase (aldose reductase), carboxyl reductase (carbonyl reductase), etc. The function of aldehyde ketone reductase is to convert carboxyl-containing substrates including steroid hormones or prostaglandins into alcohol derivatives [6,7,8]; sugar and acetaldehyde into primary alcohols; ketones into secondary alcohols, Carry out a series of metabolism and detoxification [9], including endogenous substrates, xenobiotics such as drugs, carcinogens, polycyclic aromatic hydrocarbons (PBH), etc. [10]. Microbial aldehyde ketone reductase has the effect of biotrnasfomation of harmful substances in the environment [11].

This experiment took the NADPH-dependent alpha-keto amide reductase (α-KAR) in Pichia pastoris GS115 as the research object, constructed a prokaryotic expression system, optimized its expression conditions, and used nickel Column affinity chromatography realizes the separation and purification of the target protein, and obtains a large amount of soluble enzyme protein. The experimental results in this article will provide an experimental basis for subsequent research on α-KAR.

2. Materials and Methods

2.1 Synthesis of α-KAR gene

Obtain the full sequence of AKR7A3 from GENE BANK, select the corresponding restriction site, and synthesize and optimize the α-KAR-pET28 plasmid by Shanghai Jierui Bioengineering Co., Ltd.

2.2 Strains and reagents

Escherichia coli expression strain BL21 (DE3) was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd.; IPTG (isopropyl thiogalactoside) was prepared at a concentration of 100 mg/mL for use. Low relative molecular mass protein Marker and SDS-PAGE reagents were purchased from TaKaRa (imported sub-packaging); other reagents are domestic analytical reagents.

2.3 Prokaryotic expression of AKR7A3

The recombinant plasmids a-KAR-pET28 and pET28 empty vectors were transferred into E. coli expression strain BL21 (DE3), respectively for induction and expression, cultured at 37℃ to an OD 600 of approximately 0.6, and induced by IPTG for 6 hours. After the cells were centrifuged, the supernatant was discarded, and the obtained cell pellets were resuspended in 100μL PBS and then separated and purified. The protein samples were added to 100μL of 2×SDS PAGE loading buffer, treated in boiling water for 5 minutes, and centrifuged at 12000 rpm/min, then 20μL of supernatant was taken for SDS polyacrylamide gel electrophoresis. The pET28 empty vector was used as a control to identify the expression of α-KAR.

2.4 The influence of temperature on the expression of recombinant bacteria

Keep the rotation speed of 180 rpm, the induction time of 6 hours, and the final concentration of the inducer at 1 mM, and set the culture temperature to 37℃, 30℃, 24℃, and 16℃, respectively. The culture broth was collected, ultrasonically broken, and the supernatant obtained was subjected to SDS-PAGE electrophoresis detection.

2.5 The effect of inducer concentration on the expression of recombinant bacteria

Keep the temperature at 37℃, rotate at 180rpm, the final concentration of inducer is 1mM, and set the expression time to 5h, 10h, 16h, 20h, respectively. The bacterial liquid was collected, sonicated, centrifuged and the supernatant was used for SDS-PAGE electrophoresis detection.

2.6 The influence of the induced concentration on the expression of recombinant bacteria

Keep the temperature at 37℃, rotate at 180rpm, and induce the time of 6h unchanged. The final concentration of inducer is 0.401mM, 0.60mM, 0.80mM, 1.00mM, respectively, for expression. The cells were collected, sonicated, and centrifuged, and the supernatant was subjected to SDS-PAGE electrophoresis detection.

2.7 The influence of culture speed on the expression of recombinant bacteria

The temperature was kept at 37℃, the induction time was 6h, the final concentration of the inducer was 1mM, and the speed was 110rpm, 130rpm, 150rpm, and 180rpm for expression. The cells were collected, sonicated, and centrifuged, and the supernatant was subjected to SDS-PAGE electrophoresis detection.

2.8 Kinetic detection of aldehyde and ketone reductase α-KAR enzyme activity

2.8.1 Optimization of reaction temperature

Enzyme reaction system 200μL: (2.00mmol/L) p-nitrobenzaldehyde as substrate; (0.20mmol/L) NADPH as system coenzyme; 100mM PBS as reaction buffer solution; pH needs to be controlled at 7.0; set reaction temperature at 20℃ , 25℃, 37℃, 40℃, 50℃ and 60℃, the reaction time reaches 7 minutes, and the absorbance value is measured at 340nm after the reaction.

2.8.2 Optimization of pH

Enzyme reaction system 200μL: (2.00mmol/L) p-nitrobenzaldehyde as the substrate; (0.20mmol/L) NADPH as the system coenzyme; 100mM PBS as the reaction buffer solution; the reaction temperature is set to 37℃; the pH is set It is 5.7, 6.0, 6.5, 7.0 and 8.0, the reaction time reaches 7 minutes, and the absorbance value is measured at 340nm after the reaction.

2.8.3 Optimization of Coenzyme NADPH Concentration

Enzyme reaction system 200μL: (2.00mmol/L) p-nitrobenzaldehyde as substrate; 100mM PBS as reaction buffer solution; reaction temperature is set to 37℃; pH is set to 7.0; NADPH coenzyme concentration is set to 0.05mmol/L, 0.10mmol/L, 0.15mmol/L, 0.20 mmol/L and 0.30 mmol/L, the reaction time reaches 7 minutes, and the absorbance value is measured at 340 nm after the reaction.

2.8.4 Optimization of reaction substrate concentration

Enzyme reaction system 200μL: (0.20mmol/L) NADPH as system coenzyme; 100mM PBS as reaction buffer solution; reaction temperature is set to 37℃; pH is set to 7.0; p-nitrobenzaldehyde as

substrate concentration is 0.2mmol/ L, 0.5mmol/L, 0.8mmol/L, 2mmol/L, 4mmol/L and 6mmol/L, the reaction time reaches 7 minutes, and the absorbance value is measured at 340nm after the reaction.

3. Results and analysis

3.1 Construction results of pET28a(+)-α-KAR/E.coli BL21(DE3) expression bacteria

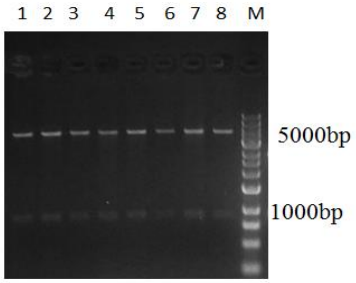

Figure 1. Plasmid identification of pET28a(+)-α-KAR/E.coli BL21(DE3). M is Marker; lanes 1-8 are the extracted plasmids

Since the pET-28(a+) vector fragment is 5422bp and the α-KAR target gene fragment is about 1000bp, this shows that the pET-28(a+)-α-KAR plasmid was successfully transferred into E.coli BL(DE3), pET28a(+)-α-KAR/E.coli BL21(DE3) expression vector has been successfully constructed and can be used in subsequent experiments.

3.2 Expression results of α-KAR protein

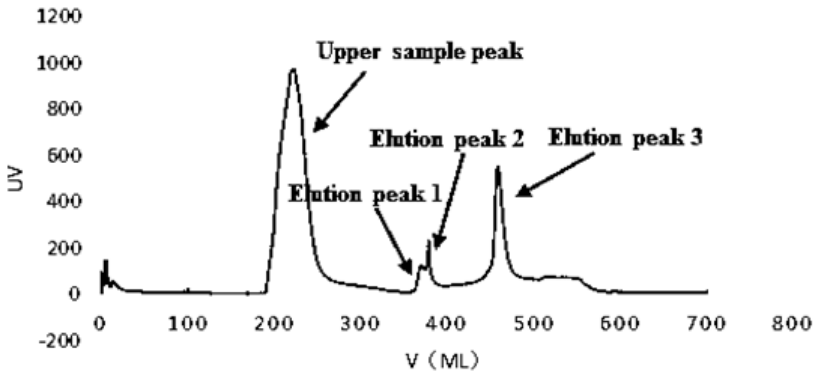

Figure 2. The affinity chromatography chromatogram of α-KAR protein nickel column

The purified components are collected and subjected to SDS-PAGE electrophoresis detection.

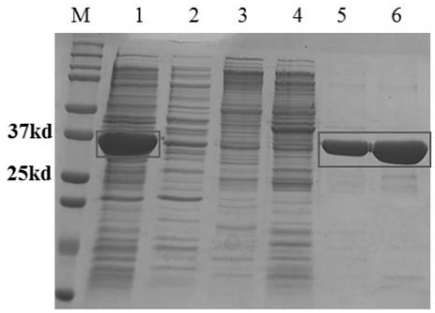

Figure 3. The result of SDS-PAGE electrophoresis

As can be seen from the figure, lane 1 is the supernatant of pET28a(+)-α-KAR/E.coli BL21(DE3) before purification, in which the target protein aldoketone reductase α-KAR is expressed in large quantities, and its size is about 37kDa. Lane 2 is the loading peak, in which there is no target protein band, indicating that the aldehyde and ketone reductase AKR7A3 is fully bound to the nickel column. Lanes 3 and 4 are the effluent after elution, and there is no target protein, indicating that it is a mixed protein peak. Lanes 5 and 6 have no contaminant protein, indicating that it is the target protein peak and is a single band.

3.3 The optimized result of expressing temperature

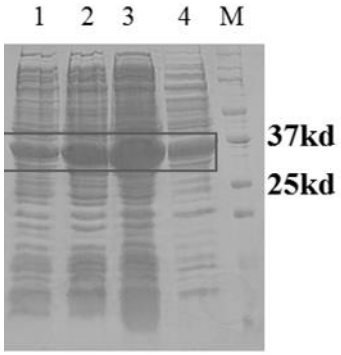

Figure 4. Result of temperature optimization. M is the protein Marker band; lanes 1-4: 16°C, 26°C, 30°C, 37°C. Soluble protein expression band under conditions;

The optimal culture temperature for pET28-α-KAR/E.coli BL21(DE3) is shown in Figure 4. It can be seen from the figure that the recombinant bacteria (pET28-α-KAR /E.coli BL21(DE3)) induce expression at 16°C, 26°C, 30°C, and 37°C, in which 3 lanes are 30°C, The expression level of the target protein in the supernatant of cell disruption is the highest, indicating that 30°C is the best induction temperature, and this condition is more suitable for the collection of the target protein.

3.4 Optimized results of inducer concentration

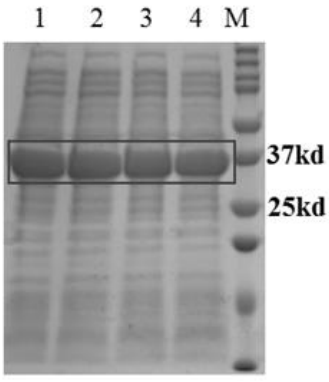

Figure 5. Result of optimization of inducer concentration. M is the protein Marker band; lanes 1-4: soluble protein expression band under the conditions of final concentration of inducer 0.40mM, 0.60mM, 0.80mM, 1.00mM

Figure 5 shows the optimized concentration of the optimal inducer IPTG for pET28-α-KAR/E.coli BL21(DE3). It can be seen from the figure that the recombinant bacteria under the conditions of the inducer IPTG concentration of 0.40mM, 0.60mM, 0.80mM, 1.00mM respectively, where 1 lane is 0.40mM, the expression of the target protein in the supernatant of the cell disruption is the largest, Indicating that the IPTG is 0.40mM is the best induction concentration.

3.5 Optimization results of induction time

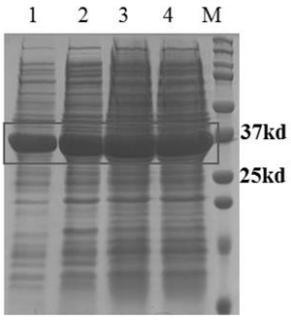

Figure 6. Optimization results of induction time. M is the protein Marker band; lanes 1-4: induction time 5h, 10h, 16h and 20h. Soluble protein expression band

Figure 6 shows the optimal induction culture time for pET28-α-KAR /E.coli BL21(DE3). It can be seen from the figure that the recombinant bacteria were induced and cultured for 5h, 10h, 16h, and 20h respectively, and the 4 lanes were 20h. The expression of the target protein in the supernatant of the cell disruption was the highest, indicating that 20h was the highest. Appropriate induction culture time.

3.6 Optimization results of culture speed

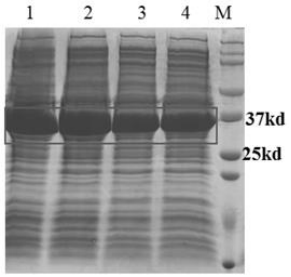

Figure 7. Result of speed optimization. M is the protein Marker band; lanes 1-4: soluble protein expression bands at 110rpm, 130rpm, 150rpm, and 180rpm, respectively

The optimized results of the optimal induction culture speed for pET28-α-KAR /E.coli BL21(DE3) are shown in Figure 7. It can be seen from the figure that the expression of the recombinant bacteria under the conditions of culture speeds of 110 rpm, 130 rpm, 150 rpm, and 180 rpm, respectively. Among them, 2 lanes are 130 rpm, and the target protein expression in the supernatant of cell disruption is the largest, indicating that 130 rpm is the best induction culture speed.

3.7 Detection of aldehyde ketone reductase α-KAR enzyme activity

From the optimization of temperature, pH, substrate, NADPH, etc., the best kinetic parameters measured are as follows:

The α-KAR enzyme activity was tested at different temperatures (20°C, 25°C, 37°C, 40°C, 50°C and 60°C), and the results showed that the enzyme activity was highest at 37°C, as shown in Figure 8(a). The α-KAR enzyme activity was tested at different pH values (5.7, 6.0, 6.5, 7.0, and 8.0), and the results showed that the enzyme activity was highest at PH=7, as shown in Figure 8(b).

The α-KAR enzyme activity was tested at different NADPH concentrations (0.05, 0.10, 0.15, 0.20L and 0.30 mmol/L), and the results showed that the enzyme activity was highest at 0.2 mmol/L, as shown in Figure 8(c). The α-KAR enzyme activity was detected at different substrate concentrations (0.2, 0.5, 0.8, 2, 4, and 6 mmol/L), and the results showed that the enzyme activity was highest at 4.00 mmol/L, as shown in Figure 8(d).

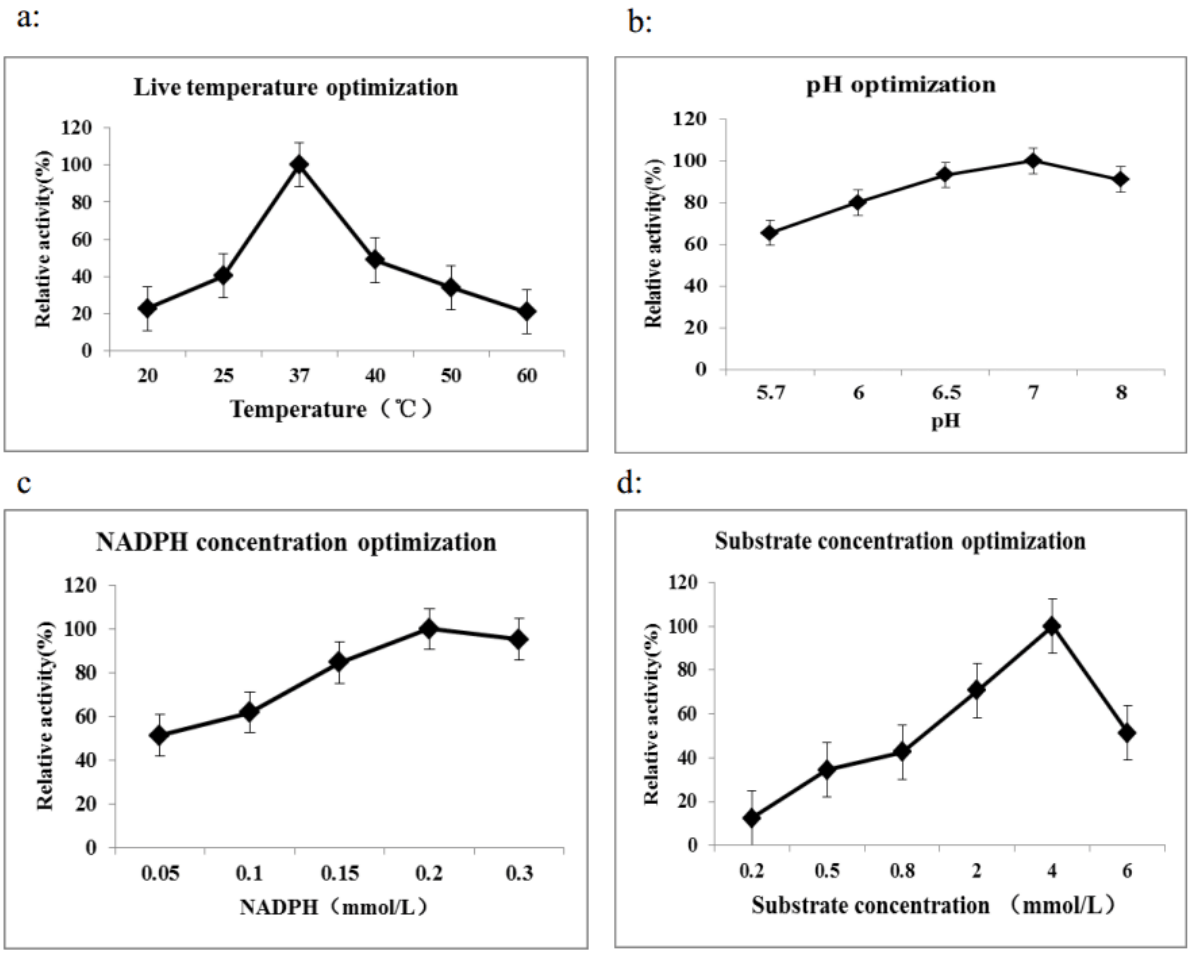

Figure 8. Determination of the optimal enzyme activity

4. Conclusion

In order to obtain the aldehyde ketone reductase α-KAR derived from Pichia pastoris and avoid the interference of Pichia pastoris expressing aldehyde ketone reductase in the eukaryotic expression system, the E. coli prokaryotic expression system was selected. Search for the gene sequence of α-KAR in GENE BANK, synthesize the optimized aldehyde ketone reductase α-KAR gene sequence, and construct the α-KAR-pET28 vector. E. coli BL21 was used as the host bacteria for expression, and the different conditions of α-KAR expression such as temperature, inducer concentration, induction time and culture speed were optimized. The optimal expression conditions obtained in the experiment were 30℃, 130rpm/h, 20h, and the inducer concentration was 0.40 mM. The optimized pET28a(+)-α-KAR/E.coli BL21(DE3) can express large amounts of proteins with good solubility, high activity and high purity. The results of enzyme activity kinetics test showed that the best enzyme activity is with p-nitrobenzaldehyde as the substrate, reaction temperature of 37℃, pH=7, NADPH concentration of 0.2mmol/L, and substrate concentration of 4.00 mmol/L.

In the future, on this basis, a large amount of α-KAR enzyme protein will be prepared by inducing engineered bacteria, which will be used in the research of the relevant redox reaction mechanism in our laboratory to provide a reliable experimental basis.

References

[1] Penning T M. Aldo-keto reductases*. Comprehensive Toxicology, 2010.

[2] Penning T M. Introduction and overview of the aldo-keto reductase superfamily. Chemical Research in Toxicology, 2004, 865(12): 3-20.

[3] Penning, Trevor M. The aldo-keto reductases (akrs): Overview. Chem Biol Interact, 2015, 234: 236-246.

[4] Zeindleberhart E, Jungblut P R, Otto A, et al. Identi-fication of tumor-associated protein variants during rat hepatocarcinogenesis. Aldose reductase. Jour-nal of Biological Chemistry, 1994, 269 (20): 14589-14594.

[5] Penning T M. The aldo-keto reductases(AKRs):overview.Chem Biol Interact,2015,234:236-246.

[6] Pundir, Seema, Jakhar, et al. Determination of urea with special emphasis on biosensors: A review. Biosensors & Bioelectronics, 2018.

[7] Evtugyn G, Hianik T. Electrochemical immuno- and aptasensors for mycotoxin determination[J]. Chemosensors, 2019, 7(1).

[8] Peltomaa R, Glahn-Martínez B, Benito-Peña E, et al. Optical biosensors for label-free detection of small molecules. Sensors, 2018, 18(12).

[9] Azzarello J T, Lin H K, Gherezghiher A, et al. Expression of AKR1C3 in renal cell carcinoma, papillary urothelial carcinoma,and Wilms' tumor. International Journal of Clinical & Experimental Pathology, 2010,3(2):147-155.

[10] Kamitori S, Iguchi A, Ohtaki A, et al. X-ray structures of nadph-dependent carbonyl reductase from sporobolomyces salmonicolor provide insights into stereoselective reductions of carbonyl compounds. Journal of Molecular Biology, 2005, 352(3): 551-558.

[11] Bodreddigari S, Jones L K, Egner P A, et al. Pro-tection against aflatoxin B1-induced cytotoxicity by expression of the cloned aflatoxin B1-aldehyde reductases rat AKR7A1 and human AKR7A3. Chemical Research in Toxicology,2008,21(5):1134-1142.